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PRIORITY DOCUMENT
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A METHOD FOR RELIEVING OR CURING SYMPTOMS OR DISEASES WHICH ARE CAUSED BY ESTROGEN DEFICIENCY OR WHICH CAN BE RELIEVED OR CURED BY ADMINISTRATION OF STEROIDAL ESTROGEN

BRIEF DESCRIPTION OF THE INVENTION

The present invention relates to a method for relieving or curing symptoms or diseases which are caused by estrogen deficiency or which can be relieved or cured by administration of steriodal estrogen, in mammals, in particular female mammals, in particular women, who suffer from breast cancer, or have a risk of recurrent breast cancer, or have a high risk of developing breast cancer.

In women cessation of ovarian function is associated with various somatic and psychological disorders which are summarized as "menopausal symptoms". The most characteristic and frequent symptoms are cessation of menstrual bleeding, hot flushes, depression, nervousness and insomnia. In addition, many women will suffer from osteoporosis due to insufficient endogenous estrogen production. Menopausal symptoms are the result of substantially reduced steroid production of the ovaries. Thus, one possibility for treatment of menopausal symptoms is substitutional treatment with ovarian hormones. Steroidal estrogens have been the treatment of choice, even though it is well known that this type of treatment may increase the risk of cancer development. In particular, in patients with a prior or a current breast cancer, it is advised not to use steroidal estrogens due the risk of stimulating "dormant" cancer cells.

An alternative to steroidal estrogens for the treatment of menopausal symptoms are plant estrogens (phytoestrogens). Among these, *Cimicifuga Racemosa* has been reported to be a successful therapeutic approach with beneficial effects on menopausal symptoms (1, 12-15, 20, 21). However, theoretically phytoestrogens would bind to estrogen receptors in target tissues including breast cancer cells in order to exert their estrogenic activity. It is therefore to be expected that phytoestrogens would stimulate growth of estrogen sensitive breast cancer cells. In support of a growth stimulatory effect of plant estrogens on breast cancer cells is the

demonstration that plant estrogens may interact with the estrogen receptors in human breast cancer cells in culture and thereby stimulate the growth of the cells (16). It would thus appear unadvisable to offer these compounds to women with a history of breast cancer or with a high risk of developing this disease.

A large fraction of women with early stage breast cancer (stage 1 and stage 2) experience cure of their disease after initial surgical treatment. It is, however, difficult to clearly define which women are most likely to have their disease return. Approximately 70% of all primary breast cancers express estrogen receptors and are therefore potentially estrogen sensitive and dependent, i.e. the growth of the cancer cells is stimulated by or dependent on estrogens (17, 18). It is therefore routinely recommended that these patients do not use steroidal estrogenic compounds at any time after the surgical resection of the primary breast cancer.

Currently available steroidal estrogens, which are most often used in hormone replacement therapy, do not discriminate between tissues or cell types. Thus, steroidal estrogens being used in clinical practice today may, while they provide a positive effect on estrogen deficiency symptoms and diseases, also carry the potential of enhancing cancer risk in women since they can stimulate cellular proliferation in several types of tissues, most notably breast tissue. Women with a family history of breast cancer and thereby with increased risk of developing breast cancer are therefore also advised not to use steroidal estrogenic compounds.

Thus, there is at present an unmet need for substances or compositions with a differential estrogenic effect, i.e. compounds which by their estrogenic effects would relieve menopausal symptoms and other estrogen deficiency-related symptoms, but without stimulatory effect on the development and/or growth of breast cancer.

If an estrogenic substance or composition could be identified which does exert estrogenic effects on menopausal symptoms and other symptoms or diseases caused by estrogen deficiency or relievable or curable by administation of steroidal estrogens, but without any influence on the growth of cancer cells, even estrogen-sensitive cancer cells, this substance or composition might find wide-spread use as a safe drug in hormone substitution therapy in

women with a history of breast cancer, women with current breast cancer, and in women with a high risk of developing breast cancer.

The present invention is based on the finding that a particular composition or material, derived from a plant, which is known to exert a valuable estrogen-like effect, is free from growth supporting effect on cancer cells and thus can be safely used by women with a history of breast cancer, women with current breast cancer, and in women with a high risk of developing breast cancer.

In one aspect, the treatment according to the invention is based on extracts of *Cimicifuga Racemosa* (CR), which have known beneficial effects on menopausal problems in women (1, 12-15, 20, 21). Such extracts may be water soluble or substantially water soluble extracts made, e.g., by alcohol extraction of *Cimicifuga Racemosa* material, in particular such as root stem. The estrogen-like effekt of CR is further documentet by the expected chances in gonadotropins following CR treatment (12, 14).

Thus, based on the findings according to the invention, women who either suffer from breast cancer, or women who have suffered from breast cancer, and who in addition are suffering from menopausal symptoms, may due to the lack of estrogenic effects on breast cancer cells choose to use CR to relief their complains. Likewise, women who have a high risk of developing breast cancer due to a family history of breast cancer and/or due to carcinoma *in situ* lesions in their breast tissue, may choose to use CR to relief their menopausal symptoms.

Cimicifuga Racemosa extract and certain substances which are found, *inter alia*, in *Cimicifuga Racemosa* extract, have been suggested as estrogenic principles also in the patent literature, confer, e.g., US Patent No. 5,830,887, WO 98/50026 and EP 0847755.

The invention is specifically based on the novel finding that CR extract shows a unique combination of a very pronounced estrogenic effect, as assessed by support of uterus growth, and, in doses which give a maximum increase in uterus growth comparable to the best increases obtainable by treatment with estradiol, shows a lack of estrogenic effects of CR on

breast cancer cells, hormone-sensitive and -dependent as well as hormone-resistant and -independent breast cancer cells. At present, it is not known whether this very beneficial combination is ascribable to one substance among the substances contained in the extract (some of which, such as formononetin, have been identified), but it seems likely that the effect is the effect of combinations of several substances in the extract, although it cannot be ruled out that it will be possible, using the principles for screening etc. which constitute an aspect of the present invention, to identify a single known or novel compound capable of exerting the valuable tissue-selective estrogenic effect.

The present invention is based on in vivo investigations performed on human breast cancer xenografts in immune deficient nude mice. Numerous studies have demonstrated that the responses of these breast cancer xenografts to endocrine treatment, i.e. ablative, inhibitive, additive, and competitive treatment are comparable to those obtained with these treatment modalities in breast cancer patients (2, 6-10). It is thus justified to presume that the response to CR of breast cancer *in situ* is comparable to the response obtained in the xenografts.

As indicated above, the findings according to the invention and the models developed by the inventors for assessing the suitability of a composition or a compound as a tissue-selective estrogenic substance open up the possibility of finding numerous other substances, compositions or chemical compounds than CR which show the valuable tissue-selective estrogenic effect.

Thus, in one aspect, the invention relates to a method for relieving or curing symptoms or diseases which are caused by estrogen deficiency, or which can be relieved or cured by administration of steroidal estrogen, in a mammal who suffers from breast cancer, or has a risk of recurrent breast cancer, or has a high risk of developing breast cancer,
the method comprising administering, to the mammal, a composition

- which has an estrogen-like effect, as evidenced by a capability of the composition of inducing uterine growth in an adult ovariectomized female rodent, and
- which is free from interaction with breast cancer cells, in particular free from stimulating effect on breast cancer ,

thereby treating the estrogen deficiency-conditioned symptom or disease without introducing a risk of provoking the development of clinically evident breast cancer and/or stimulating growth of existing breast cancer cells in the mammal.

This means treatment of the symptoms or diseases can now be performed without introducing the risk of stimulating breast cancer cells which is associated with administration of steroidal estrogen to a female mammal who suffers from breast cancer, or has a risk of recurrent breast cancer, or has a high risk of developing breast cancer.

While by far the most important patient group to receive the treatment will be women, it is contemplated that the method of the invention may also be useful in animal treatment, such as for dogs, and it is also known that men may suffer from breast cancer and at the same time from symptoms or diseases which can be treated using estrogen.

The estrogen-like effect possessed by the composition manifests itself in the composition being capable of inducing an increase in uterine weight in adult ovariectomized NMRI female athymic nude mice, and for preferred composiitons, the increase in uterine weight following a dose comparable to a normal dose for the mammal to be treated may correspond to a weight increase seen in the same test animal following estradiol treatment, which will often be substantially the maximum weight increase obtainable in the same test animal by estrogen treatment. It is, of course, also possible to measure estrogenic effect manifested in stimulation of uterus cells in, e.g., vaginal smear from women.

It is an important aspect of the invention that the composition is one which has substantially no effect on the growth of breast cancer cells, neither a positive, agonizing, effect, nor a negative, antagonizing, effect, and that this applies both for estrogen receptor-negative breast cancer cells (and also includes lack of indirect effect) and for estrogen receptor-positive breast cancer cells.

Specific principles for assessing the presence of the desired estrogenic effect and the lack of the undesired effect on the growth of breast cancer cells are emphasized in the claims and are explained in deltail in the detailed section which follows.

In addition to the above-mentioned menopausal symptoms, the estrogen deficiency-conditioned symptom or disease may be osteoporosis, osteochondrosis, hyperlipidaemia, hypercholesterolaemia, or arteriosclerosis.

The composition may, in a presently preferred embodiment, be a composition containing substances contained in *Cimicifuga Racemosa* extract, or derivatives thereof. Thus, the composition may be or may contain *Cimicifuga Racemosa* extract (either in a liquid form or in the dry form used in the examples. Doses of the extract may be from, e.g. 1-2 mg daily to 10-20 mg daily on the basis of the extract, such as 2-3 mg twice daily for a woman, and doses of other active principles than the CR extract can be assessed by the person skilled in the art based on the relative activity compared to CR extract, or based on the experiments explained in the following.

The composition to be used according to the invention may be an oral composition or a composition suitable for any other appropriate route of administration, and examples of suitable pharmaceutical preparations can, e.g., be found in the literature, including the above-mentioned patent literature.

The composition may also be a composition comprising *Cimicifuga Racemosa* plant parts, or it may be a composition containing one or more chemical compounds contained in *Cimicifuga Racemosa* extract, or derivatives thereof.

However, as mentioned above, based on the screening made possible through the present invention, other substances or compositions showing the valuable tissue-selective estrogenic effect may be identified.

Thus, another aspect of the invention relates to a method for screening for substances or compositions which can be used in the method according to claim 1, comprising subjecting test substances or compositions to

1) testing for possible estrogen-like effect in normal tissue by measuring increase in uterine

weight in an adult ovariectomized female rodent and
2) testing for possible estrogenic effect in breast cancer,
and selecting, as candidates for tissue-selective estrogenic substances or compositions useful in the method according to claim 1, substances or compositions which,
a) are capable of inducing uterine growth in an adult ovariectomized female rodent, and at the same time
b) have no effect on the growth of estrogen receptor-negative cancer cells and no effect on estrogen receptor-positive cancer cells in the doses in which they induce uterine growth.

This aspect of the invention is also explained in greater detail in the following, as are protocols for clinical work.

DETAILED DESCRIPTION OF THE INVENTION

Ovariectomized rodents are suitable animal models for the study of estrogenic effects (2, 6-11). Administration of steroidal estrogens or other estrogenic compounds will result in increased uterine tissue weight. By using immune deficient rodents, the effects of steroidal estrogens and other estrogenic compounds can be studied simultaneously in the uterine tissue and in xenotransplanted human tumours.

In Example 1 the estrogenic effect of a CR composition in the form of CR extract on mouse uterine tissue is described. The dose selected was based on the recommended daily intake of CR extract in women, which is 2.4 mg/kg twice daily.

The increase in uterine weight induced by CR extract corresponds to that obtained following treatment with estradiol. Thus, it can be concluded that CR has estrogen-like effect on murine uterine tissue and that the applied administration and dose of CR is sufficient to induce this effect.

Example 2 describes the effect of CR extract on tumour development and growth of an estrogen receptor-negative human breast cancer grown in nude mice. Using the same dose of

CR extract which induced increase in uterine weight, no effect was observed on the human breast cancer xenograft.

In Example 3, the estrogen receptor-positive human breast cancer cell line MCF-7 was inoculated into nude mice and treated with estradiol and CR extract. The CR extract dose was either the same as used in Example 1 and 2 or a 10 fold higher dose. The MCF-7 human breast cancer only forms tumours in ovariectomized or intact female nude mice in the presence of estrogen supplementation. In accordance with this, estrogen supplementation resulted in the development of growing tumours, while untreated mice did not develop any tumours. Treatment with CR extract in both dose groups did not result in tumour development in any of the mice studied. These results suggest that CR extract has no estrogenic effect on estrogen receptor-positive breast cancer cells.

Example 4 describes the effect of orally administered CR extract and subcutaneously administered estradiol on tumour development and tumour growth of the estrogen receptor-positive and estrogen dependent MCF-7 human breast cancer xenograft in nude mice. The CR extract dose was 100 fold higher than the recommended daily dose for humans. The treatment with CR extract and estradiol was given alone and in combination. CR extract had no growth supportive effect or growth inhibitory effect whether given alone or in combination with E2, suggesting that CR extract has neither potentiating nor inhibitory effect on estrogen sensitive breast cancer cells. The results further imply that CR extract does not bind to estrogen receptors of the MCF-7 cells, in that it does not antagonize the growth stimulatory effect of estradiol.

Example 5 describes an experimental model system for the testing of compositions or compounds ("drugs") with putative differential estrogen-like effects in normal tissues and in breast cancer. Possible estrogen-like effects in normal tissues are tested by measuring increase in mouse uterine weight as the end point, whereas possible estrogenic effect in breast cancer is tested by measuring growth supportive effect in the estrogen sensitive MCF-7 breast cancer xenograft as the end point. The tests can be performed in the same mice or in identical mice.

Example 6 describes a clinical protocol for safety studies of CR treatment in women with advanced breast cancer. Patients are randomized to treatment with CR or placebo and will be followed until death. Survival curves for each group will be constructed in order to determine the possible effect of CR in this patient population. It will be understood that this protocol can be adapted for use for safety studies of treatment with any composition, CR-based or not, which fulfils the criteria for being useful in the method according to the invention.

Example 7 describes a clinical protocol for safety studies of CR treatment in women with advanced breast cancer and who have obtained a complete or partial response upon conventional antineoplastic therapy. Patients are randomized to treatment with CR or placebo and will be followed until death. Time to progression in each treatment group will be determined and compared in order to establish whether CR has any effect on cancer progression in this patient population. Again, it will be understood that this protocol can be adapted for use for safety studies of treatment with any composition, CR-based or not, which fulfils the criteria for being useful in the method according to the invention.

In example 8, a clinical protocol is described for safety studies of CR treatment in women with a prior diagnosis of breast cancer and with no indication of recurrence. Patients are randomized to treatment with CR or placebo and will be followed for 5 years. Univariate recurrence-free and overall survival curves will be constructed and compared in order to establish whether CR has any effect on these two parameters. Also this protocol can be adapted for use for safety studies of treatment with any composition, CR-based or not, which fulfils the criteria for being useful in the method according to the invention.

Example 9 describes clinical CR extract treatment of estrogen deficiency conditions other than menopausal symptoms in women with current breast cancer, with a prior diagnosis of breast cancer, and with an increased risk of developing breast cancer. The diseases include osteoporosis, osteochondrosis, hyperlipidaemia, hypercholesterolaemia, arteriosclerosis and other conditions which can be cured or relieved by estrogen replacement therapy. It will be understood that the principles which appear from this example can be used with any other composition which fulfils

Example 1

EFFECT OF ORALLY ADMINISTERED CR ON UTERINE WEIGHT IN MICE

This example describes the effect of orally administered CR on uterine weight in adult ovariectomized NMRI female athymic nude mice.

MATERIALS AND METHODS

Ovariectomy, randomization and identification

After acclimatization the animals were ovariectomized and randomized to two treatment groups and a group of untreated controls (see below). The animals and cages were marked accordingly.

Study design

The mice were ovariectomized and randomized at day 0. One group of mice served as untreated controls (n=5), and one group of mice (n=5) was given estradiol from day 1 to 8 by insertion of a 0.72 mg subcutaneous E2 pellet. In the last group the mice (n=6) were given CR extract orally twice daily from day 1 to 8.

Observations

The animals were observed daily during the experimental period of 8 days.

CR dosing procedure

Each day a new suspension/solution of 1.0 mg CR composition in the form of root stem extract (Biona A/S, Denmark) in 16.6 ml sterile water was prepared and kept at +4°C. Dosing was performed orally with a dosing volume of 0.1 ml to each mouse twice daily. Each mouse in the CR group thus received 0.48 mg CR extract/kg/day.

Estradiol (E2) treatment procedure

One day after ovariectomy, a 0.72 mg estradiol slow release pellet (Innovative Research) was inoculated subcutaneously in the neck of the mice using a trocar.

Termination of experiment

The experiment was terminated at day 8 after ovariectomy. All animals were sacrificed by cervical dislocation and the mouse uterine weights were determined.

RESULTS

The calculated mean uterine weights and the corresponding ranges were 0.08 g [0.05 - 0.11], 0.13 g [0.11 - 0.15], and 0.14 g [0.11 - 0.21] in the control, estradiol and CR groups, respectively (*Table 1* and *Figure 1*).

DISCUSSION

The CR extract dose of 0.48 mg/kg/day was chosen to simulate the daily intake of CR extract recommended for human beings (Biona A/S, Denmark).

CR extract had an *in vivo* effect on mouse uterine weight which was comparable to that seen following E2 treatment.

Example 2

EFFECT OF ORALLY ADMINISTERED CR EXTRACT ON TUMOUR DEVELOPMENT AND GROWTH OF THE MDA-MB-231 HUMAN BREAST CANCER XENOGRAFT

This example describes the effect of orally administered CR extract on tumour development and tumour growth of the estrogen receptor-negative and estrogen resistant and independent MDA-MB-231 human breast cancer xenograft in nude mice (4).

MATERIALS AND METHODS

Cells

The human mammary cancer cell line MDA-MB-231 was used. Near confluent *in vitro* grown cells were harvested using a cell scraper. After centrifugation an aliquot of the cells was stained by Trypan Blue and counted. The cells were resuspended in fresh medium to a final concentration of 1 x 10^6 viable cells per inoculum (0.2 ml).

Animals

The experiment was performed in a total of 21 six-week-old female META/Bom™ athymic nude mice. An acclimatization period of one week was allowed in order to exclude animals in poor condition.

The mice were kept under sterile conditions in laminar air flow clean benches. Type III Macrolon cages (42 x 26 x 15 cm) with five animals in each cage was used. The mice were allowed sterile water and food pellets *ad libitum*.
The cages and the bedding were changed once a week. The room temperature was 25 ± 2^0C, and the relative humidity 55 ± 5%. The room was illuminated 24 hours a day.

Growth of MDA-MB-231

Animal randomization and identification

After acclimatization, the animals were randomized to a group of 11 treated animals and a group of 10 control mice. Each animal was identified by earmarks and each cage was marked to identify group and animal earmarks.

Study design

At day 0 the animals were inoculated with tumour cells subcutaneously in both flanks.
The mice were then randomized into either CR extract treatment or treatment with sterile water. The mice of the CR extract treatment group were given CR extract orally twice daily from the day of cell inoculation and until termination of the experiment. Control mice received sterile water twice daily.

CR extract dosing procedure

Each day a new suspension of 2.0 mg CR extract (Biona A/S, Denmark) in 16.6 ml sterile water was prepared and kept at +4°C. Dosing was performed orally with a dosing volume of 0.05 ml to each mouse twice daily. Mice in the control group received 0.05 ml sterile water orally twice daily. Each mouse in the CR group thus received 0.48 mg CR extract/kg/day.

Observations

The animals were observed daily during the experimental period of 38 days.

When the tumours became measurable they were measured in two dimensions three times a week using a sliding gauge. Tumour growth curves were constructed and growth curve parameters were calculated (19).

Tumours that did not grow during the entire experimental period and tumour inocula without take were excluded from the analysis.

Termination of experiment

The experiment was terminated when treated tumours had shown increasing size during at least six consecutive growth recordings. At termination of the experiment, the animals were sacrificed by cervical dislocation.

RESULTS

The CR treatment was not toxic to the animals.

When this experiment was terminated, a total of 31 tumours in 17 mice were evaluable. 15 tumours developed in control mice and 16 tumours developed in the CR treated mice. *Figures 2* show the mean tumour area growth curves constructed from the individual tumour measurements. Calculated tumour growth curve parameters are listed in *Table 2*.

The specific growth rate, α (*Table 2*) of CR treated tumours was 0.0144 compared to 0.0165 of the vehicle treated control tumours, and as a consequence the calculated tumour volume

doubling times (TD) were 14.9 days and 13.0 days of the CR treated and control tumours, respectively.

DISCUSSION

The results of the growth curve analysis indicate that the applied dose of CR extract had no effect on growth of the estrogen and progesterone receptor negative MDA-MB-231 human breast cancer cell line. Similar experiments as those described in this example can be performed using other estrogen receptor-negative human breast cancer cell lines, e.g. MDA-MB-435.

Example 3

EFFECT OF ORALLY ADMINISTERED CR extract ON TUMOUR DEVELOPMENT AND GROWTH OF THE MCF-7 HUMAN BREAST CANCER XENOGRAFT

This example describes the effect of orally administered CR on tumour development and tumour growth of the estrogen receptor-positive and estrogen dependent MCF-7 human breast cancer xenograft in nude mice (4).

MATERIALS AND METHODS

Cells

The estrogen and progesterone receptor positive human mammary cancer cell line MCF-7 was used.

Near confluent *in vitro* grown cells were harvested using a cell scraper. After centrifugation an aliquot of the cells was stained by Trypan Blue and counted to determine the fraction of viable cells. The cells were resuspended in fresh medium to a final concentration of 10^6 - 10^7 viable cells per inoculum (0.1-0.2 ml). The cells were inoculated subcutaneously into both flanks of intact or ovariectomized female nude mice.

In separate experiments, xenotransplanted tumours in intact female nude mice were used as the source for the experiments. The tumours were excised under general anaesthesia with

Ketalar[7]/Rompun[7] and 1-2 mm diameter tumour blocks were prepared and inoculated into the right flank of recipient mice.

Animals

The experiment was performed in a total of 80 six-week-old female NMRI/Bom™ athymic nude mice. An acclimatization period of one week was allowed in order to exclude animals in poor condition.

After acclimatization some of the animals were ovariectomized under general anaesthesia and using standard procedures. Transplantation of tumours and inoculation of cells were performed following at least one week to ensure full recovery after the ovariectomy.

The mice were kept under sterile conditions in laminar air flow clean benches. Type III Macrolon cages (42 x 26 x 15 cm) with five animals in each cage were used. The mice were allowed sterile water and food pellets *ad libitum*.
The cages and the bedding were changed once a week. The room temperature was 25 ± 2°C, and the relative humidity 55 ± 5%. The room was illuminated 24 hours a day.

Growth of MCF-7

Animal randomization and identification

After inoculation of cells or tumour blocks the animals were randomized into treatment groups according to the study design. Each animal was identified by earmarks and each cage was marked to identify group and animal earmarks.

Study design

Three series of experiments were performed.

Study 1 Forty ovariectomized animals were inoculated with 10^6 MCF-7 cells, and the mice were randomized into four treatment groups: Untreated controls, 0.48 mg/kg/day CR extract, 4.8 mg/kg/day CR extract, and 0.72 mg estradiol slow release pellet (Innovative Research).

Study 2 Twenty intact female nude mice were inoculated with 10^7 MCF-7 cells, and

the mice were randomized into two treatment groups: 4.8 mg/kg/day CR extract or 0.72 mg estradiol slow release pellet (Innovative Research).

Study 3 Twenty intact female nude mice were inoculated with tumour blocks of MCF-7 xenografts into the right flank of recipient mice, and the mice were randomized into two treatment groups: 4.8 mg/kg/day CR extract and 0.72 mg estradiol slow release pellet (Innovative Research).

CR treatment procedure

Each day a new suspension of 0.5 or 5.0 mg CR extract (Biona A/S, Denmark) in 4.15 ml sterile water was prepared and kept at room temperature. Dosing was performed orally with a dosing volume of 0.05 ml to each mouse twice daily corresponding to 0.48 and 4.8 mg/kg/day, respectively.

Treatment was initiated at the day of cell inoculation.

Estradiol (E2) treatment procedure

At the day of inoculation of cells or tumour blocks a 0.72 mg estradiol slow release pellet (Innovative Research) was inoculated subcutaneously in the neck of the mice using a trocar.

Observation & calculations

The animals were observed daily during the experimental periods. When the tumours became measurable, they were measured in two dimensions three times a week using a sliding gauge. Tumour growth curves were constructed and growth curve parameters were calculated (19).

Termination of experiment

The experiment was terminated when growing tumours (only E2 stimulated tumours) had shown increase in size during at least six consecutive growth recordings. At termination of the experiment, the animals were sacrificed by cervical dislocation.

RESULTS

In non of the three studies toxicity was observed in mice treated with CR extract.

Study 1. The experiment was terminated 27 days after initiation because of a very low take rate. No tumours developed in the group of untreated controls or in the two CR extract treatment groups. However, only in one of the 10 E2 treated mice a growing tumour appeared.

Study 2. In this experiment six out of 9 evaluable E2 treated mice developed a total of 10 growing MCF-7 tumours. In contrast, in 10 out of 10 evaluable CR treated animals no tumours developed.

Study 3. When this experiment was terminated 52 days after transplantation, tumours had developed in 7 out of 10 mice in the E2 treatment group. No tumours were detected in 10 mice treated with CR.

Figure 3 shows the mean tumour area growth curve constructed from the individual tumour measurements of the E2 treated tumours, and the lack of tumour growth in CR treated mice is indicated in the figure. Calculated tumour growth curve parameters are listed in *Table 3.*

DISCUSSION

The presented series of experiments showed that the applied CR treatment did not have tumour stimulatory effect on estrogen dependent MCF-7 human breast carcinoma xenografts. In contrast , treatment with E2 supported the growth of this tumour, thus confirming the ability of MCF-7 to form tumours in nude mice under adequate hormonal substitution. Similar experiments as those described in this example can be performed using other estrogen receptor-positive human breast cancer cell lines, e.g. T47D and ZR75-1 (5).

The results of *Study 2* and *3* were identical in the observed tumour growth despite the difference in method of tumour establishment, i.e. *in vitro* grown single cell suspensions *vs.* solid tumour blocks.

Although tumour growth was only observed in the E2 treatment group of *Study 1*, the results of this experiment was inconclusive due to the low take rate. This was probably caused by a

relatively low cell dose of the inocula (10^6 cells). Therefore the cell dose was increased to 10^7 cells/inoculum in *Study 2*, resulting in a take rate comparable to that obtained with transplantation of tumour blocks in *Study 3*.

The CR extract dose of 0:48 mg/kg/day was chosen to simulate the daily intake of CR extract recommended for human beings (Biona A/S, Demark). The results showed that even a 10-fold increase in the dose of CR extract did not support the growth of MCF-7.

The MCF-7 breast carcinoma in nude mice is a slow growing tumour (3). The specific growth rate, $\alpha = 0.0076$ (*Table 3*) corresponds to a tumour volume doubling time (TD) of 28.3 days.

Example 4

EFFECT OF CONCOMITANT ORAL CR AND SUBCUTANEOUS ESTROGEN TREATMENT ON TUMOUR DEVELOPMENT AND TUMOUR GROWTH

This example describes the effect of orally administered CR and subcutaneously administered estrogen on tumour development and tumour growth of the estrogen receptor-positive and estrogen dependent MCF-7 human breast cancer xenograft in nude mice (4).

MATERIALS AND METHODS

Cells

The estrogen and progesterone receptor positive human mammary cancer cell line MCF-7 was used.

Near confluent *in vitro* grown cells were harvested using a cell scraper. After centrifugation an aliquot of the cells was stained by Trypan Blue and counted to determine the fraction of viable cells. The cells were resuspended in fresh medium to a final concentration of 10^6 - 10^7 viable cells per inoculum (0.1-0.2 ml). The cells were inoculated subcutaneously into both flanks of intact female nude mice. One resulting MCF-7 tumour was used for serial transplantation into all the mice of this study.

Animals

The experiment was performed in a total of 32 six-week-old female NMRI/Bom™ athymic nude mice. An acclimatization period of one week was allowed in order to exclude animals in poor condition.

The mice were kept under sterile conditions in laminar air flow clean benches. The mice were allowed sterile water and food pellets *ad libitum*. The cages and the bedding were changed once a week. The room temperature was 25 ± 2°C, and the relative humidity 55 ± 5%.

Growth of MCF-7

Animal randomization and identification

After inoculation of tumour blocks the animals were randomized into treatment groups according to the study design. Each animal was identified by earmarks and each cage was marked to identify group and animal earmarks.

Study design

Thirty-two intact female nude mice were transplanted with approximately 1-mm-diameter blocks of MCF-7 tumour into the both flanks of recipient mice, and the mice were randomized into four treatment groups:

1: 48 mg/kg/day CR extract orally.

2: 0.72 mg slow release estradiol pellet s.c. (Innovative Research).

3: 48 mg/kg/day CR extract and 0.72 mg estradiol slow release pellet (Innovative Research).

4: Sterile water p.o. twice daily.

CR dosing procedure

Each day a new suspension of 50 mg CR extract (Biona A/S, Denmark) in 4.15 ml sterile water was prepared and kept at room temperature. Dosing was performed orally with a dosing volume of 0.05 ml to each mouse twice daily corresponding to 48 mg/kg/day. Treatment was initiated at the day of tumour tissue inoculation.

Estradiol (E2) treatment procedure

At the day of inoculation of tumour blocks a 0.72 mg estradiol slow release pellet (Innovative Research) was inoculated subcutaneously in the neck of the mice using a trocar.

Observation & calculations

The animals were observed daily during the experimental period. When the tumours became measurable, they were measured in two dimensions three time a week using a sliding gauge. Tumour growth curves were constructed and growth curve parameters were calculated (19).

Termination of experiment

The experiment was terminated when the growing tumours (only in the E2 stimulated groups 3 and 4) had shown increase in size during at least six consecutive growth recordings. At termination of the experiment, the animals were sacrificed by cervical dislocation.

RESULTS

No toxicity was observed in the CR treated mice.

As expected, the untreated control tumours showed no significant growth. At day 20 after transplantation 10 persisting tumour inocula were evaluable (*Figure 4*). The CR treatment did not support the growth of the MCF-7 tumour. The mean tumour area of 5 evaluable tumour inocula at 20 days after transplantation was the same as that of the control group.

The E2 treatment had growth supportive effect on the MCF-7 tumour xenograft. In this group, 8 tumours showed growth, and at day 20 the tumours had reached a mean tumour area of 39 sq mm (*Figure 4*). The addition of CR to E2 did not influence the growth of the tumour. At 20 days after transplantation, the mean tumour area of 11 evaluable tumours in the combined treatment group were the same as that of the E2 group.

DISCUSSION

This study shows that even a 100 fold increase in the daily recommended CR dose for

humans does not induce tumours in mice inoculated with hormone receptor positive human breast cancer cells. In contrast, the positive control treatment with E2 has the expected growth supportive effect on the estrogen sensitive and dependent MCF-7 breast cancer xenograft.

In the group where mice received both CR and estrogen treatment no differences were seen when compared to the estrogen treatment only. This suggests that CR neither has a potentiating nor an inhibitory (antagonizing) effect on estrogen sensitive breast tumours. The observation further implies that CR does not bind to estrogen receptors in MCF-7 cells.

Example 5

EXPERIMENTAL MODEL FOR TESTING DIFFERENTIAL ESTROGENIC EFFECTS

This example describes an experimental model system for the investigation of drugs or combination of drugs with putative differential estrogenic effects in normal tissues and in breast cancer.

Normal tissues

The possible estrogenic effects on normal tissues are tested using increase in uterine weight as the end point. The investigation includes an estradiol treatment group which serves as a functional control of the study system.

Animals

Six-eight-week-old female NMRI/Bom™ athymic nude (*nu/nu*) mice are used. After acclimatization the mice are ovariectomized in order to avoid influence of endogenous estrogen. The mice are randomised into treatment groups and a group of untreated controls.

The experiments described in this example can be performed with other strains of mice.

Study design

The mice are ovariectomized and randomised at day 0. One group serves as untreated

controls (n=5-10), and one group (n=5-10) are given estradiol (E2) from day 1 by insertion of a 0.72 mg subcutaneous slow release E2 pellet (Innovative Research) in the neck of the mice using a trocar.

The last group(s) (n=5-10 in each group) are treated with the test drug(s) using relevant dose(s) and schedule(s).

Observations

The animals are observed daily during the experimental period of 8 days. Animals dying before termination of the experiment are excluded from the analysis.

Dosing procedure of test drug(s)

Suspensions of the drug(s) are prepared using appropriate vehicle(s). The treatment is given by the most convenient route of administration (orally, intraperitoneally, or intravenously) and with appropriate dosing volume(s).

Dose(s) of the drug(s) are selected in relevant dose range(s), and schedule(s) with expected higher efficiency are selected.

Termination of experiment

The experiment is terminated at day 8 after ovariectomy. All animals are sacrificed by cervical dislocation and the mouse uterine weights are determined.

Evaluation

The mouse uterine weight data are used to calculate mean uterine weights of the control, estradiol, and test groups, respectively.

A significant increase in mean uterine weight of the E2 group compared to the controls serves as functional control of the study system.

A significant increase in mean uterine weight of the test drug(s) treatment group(s) are considered evidence of estrogenic effect on normal tissues.

Comparison of the increase in mean uterine weight in the E2 group and test group(s) are used to semiquantitate the estrogenic effect of the investigated drug(s), dose(s), and schedule(s).

Breast cancer

The possible estrogenic effects on breast cancer cells are tested using growth stimulation of an estrogen sensitive human breast cancer xenograft as the end point.

The investigation is performed in two human breast cancer xenografts using nude mice identical to those used for the uterine weight investigation. One tumour, MDA-MB-231 is estrogen and progesterone receptor negative, and therefore estrogen resistant and independent (4). The other tumour, MCF-7 is estrogen and progesterone receptor positive, and estrogen sensitive and dependent (4). The reason for inclusion of the receptor negative breast tumour xenograft in the test system is to identify possible unspecific effects, i.e. non-estrogenic effects which are independent of binding of the drug(s) to estrogen receptors of the tumour cells.

The investigation includes estradiol treatment of the estrogen sensitive MCF-7 tumour xenografts which serves as a functional control of the study system.

Tumour cells

The human mammary cancer cell lines MDA-MB-231 and MCF-7 are used. Near confluent *in vitro* grown cells are harvested using a cell scraper. After centrifugation an aliquot of the cells is stained by Trypan Blue and counted to determine the fraction of viable cells. The cells are resuspended in fresh medium to a final concentration of 10^6 - 10^7 viable cells per inoculum (0.1-0.2 ml). The cells are inoculated subcutaneously into both flanks of ovariectomized or intact female nude mice.

Animals

Six-week-old female athymic NMRI/Bom™ nude (*nu/nu*) mice are used. An acclimatization period of one week is allowed in order to exclude animals in poor condition. The experiments described in this example can be performed with other strains of mice.

Ovariectomy is performed under general anaesthesia and using standard procedures following acclimatization of the animals.

In ovariectomized mice inoculation of tumour cells is performed after at least one week to ensure full recovery after the ovariectomy.

After inoculation of cells the animals are randomized into treatment groups according to the study design.

The mice are kept under sterile conditions in laminar air flow clean benches. The mice are allowed sterile water and food pellets *ad libitum*. The room temperature is 25 ± 2°C, and the relative humidity 55 ± 5%.

Study design

The growth experiments with the two tumours are performed in separate series using similar study design.

At day 0, the animals are inoculated with tumour cells subcutaneously in both flanks. The mice are then randomized into one group of untreated (vehicle) controls (n=10) and a group (n=10) given E2 from day 1 by insertion of a 0.72 mg subcutaneous slow release E2 pellet (Innovative Research) in the neck of the mice using a trocars. A third group(s) (n=10 in each group) is treated with test drug(s) using relevant dose(s) and schedule(s). The E2 treatment group is only included in the treatment of MCF-7 xenograft.

The dosing procedures are planned as described in the uterine weight section

The animals are observed daily during the experimental period. When the tumours become measurable they are measured in two dimensions three time a week using a sliding gauge. Tumour growth curves are constructed and growth curve parameters are calculated (19).

The experiment is terminated when growing tumours have shown increasing size during at least six consecutive growth recordings. At termination of the experiment, the animals are sacrificed by cervical dislocation.

Evaluation

Using a computer program the tumour measurements are used to construct mean growth curves and to calculate tumour growth curve parameters (19).

Stimulatory effect of E2 on the MCF-7 xenografts serves as functional control of the study system.

A growth supportive effect of the drug(s) in the MCF-7 xenograft indicates an estrogen-like effect mediated through estrogen receptors of the cancer cells. If a growth supportive effect of the drug(s) is also found in the MDA-MB-231 xenograft, this is considered evidence that the effect observed is not mediated through the estrogen receptors of the tumour cells. However, in order to be chosen as a candidate for a substance or composition useful in the method of the invention, the substance or composition should show neither growth supportive effect in the MCF-7 xenograft nor growth supportive effect (which would then be an indirect growth supportive effect) in the MDA-MB-231 xenograft.

Comparison of the growth supportive effect in the E2 group and test group(s) are used to semiquantitate the estrogenic effect of the investigated drug(s), dose(s), and schedule(s).

Similar experiments as those described in this example can be performed using other estrogen receptor-positive and negative human breast cancer cell lines, e.g. T47D, ZR75-1, and MDA-MB-435 (5).

Example 6

CLINICAL EVALUATION OF THE SAFETY OF CR TREATMENT IN WOMEN WITH ADVANCED BREAST CANCER

This example describes a clinical protocol for safety studies of CR treatment in women with advanced breast cancer.

MATERIALS AND METHODS

Patients

Advanced breast cancer patients with measurable disease will be included. The patients should suffer from menopausal symptoms. No chemotherapy or endocrine therapy should be given concomitantly with the CR treatment. Estrogen receptor (ER) status on the cancer cells should be determined.

Treatment

CR extract should be administered orally twice daily at a dose of 0.48 mg/kg daily. Treatment period should be 3 months.

Study design

Patients will be randomized to either treatment with CR extract or placebo. Patients in both treatment groups will be followed until death.

Dose(s) and schedule(s) can be subjected to changes.

Evaluation

Measurable lesions (from one to three lesions) should be defined and measured before commencement of CR extract treatment. Effect of CR on menopausal symptoms will be registered. Any site effects associated with CR treatment will be registered.

At the end of the experiment measurable lesions will be evaluated and any effect according to WHO criterias will be noted.

Survival curves for each group will be constructed and it will be determined whether CR has any effects on survival in this patient population.

Example 7

CLINICAL EVALUATION OF THE SAFETY OF TREATMENT IN WOMEN WITH ADVANCED BREAST CANCER

This example described a clinical protocol for safety studies of CR treatment in women with advanced breast cancer.

MATERIALS AND METHODS

Patients

Advanced breast cancer patients who have obtained a complete or partial response upon conventional antineoplastic therapy will be included. The patients should suffer from menopausal symptoms. No chemotherapy or endocrine therapy should be given concomitantly with the CR treatment. ER status on the cancer cells should be determined.

Treatment

CR extract should be administered orally twice daily at a dose of 0.48 mg/kg daily. Treatment period should be 3 months.

Study design

Patients will be randomized to either treatment with CR or placebo and followed until death.

Dose(s) and schedule(s) can be subjected to changes.

Evaluation

Time to progression (WHO criterias) will be determined. Effect of CR on menopausal symptoms will be registered. Any site effects associated with CR treatment will be registered.

Time to progression in each treatment group will be determined and compared between groups in order to determine whether CR has any effect on cancer progression in this patient population.

Example 8

CLINICAL EVALUATION OF THE SAFETY OF CR TREATMENT IN WOMEN WHO HAVE HAD A PRIOR BREAST CANCER

This example describes a clinical protocol for safety studies of CR treatment in women with a prior diagnosis of breast cancer and with no indication of recurrence.

MATERIALS AND METHODS

Patients

Women with a prior diagnosis of breast cancer who are suffering from menopausal symptoms will be included. The patients must have no sign of recurrent disease. No antineoplastic treatment should be given concomitantly with the CR treatment. ER status on the cancer cells should be determined.

Treatment

CR should be administered orally twice daily at a dose of 0.48 mg/kg daily. Treatment period should be 1 year.

Study design

Patients should be randomized to either treatment with CR or placebo. Patients in both groups will be followed for 5 years.

Dose(s) and schedule(s) can be subjected to changes.

Evaluation

Time to recurrence of disease will be determined. Effect of CR on menopausal symptoms will be registered. Any site effects associated with CR treatment will be registered.

Univariate recurrence-free survival and overall survival curves will be constructed for both treatment groups and compared in order to establish whether CR treatment has any effect on these two parameters.

Example 9

EFFECT OF CR TREATMENT ON ESTROGEN DEFICIENCY CONDITIONS OTHER THAN MENOPAUSAL SYMPTOMS IN PATIENTS WITH CURRENT BREAST CANCER, WITH A PRIOR DIAGNOSIS OF BREAST CANCER, AND WITH AN INCREASED RISK OF DEVELOPING BREAST CANCER

This example describes CR treatment of estrogen deficiency conditions including osteoporosis, hyperlipidaemia, arteriosclerosis *etc.* in the subgroup of women who are currently suffering from breast cancer, who have a prior history of breast cancer, and women with an

increased risk of developing breast cancer.

MATERIALS AND METHODS

Patients

Women belonging to one of the above mentioned breast cancer related groups and suffering from estrogen deficiency, will be candidates for CR treatment. Diseases related to estrogen deficiency include osteoporosis, osteochondrosis, hyperlipidaemia, hypercholesterolaemia, and arteriosclerosis.

In addition, women belonging to one of the above mentioned three breast cancer related groups and suffering from diseases which can be cured or relieved by estrogen replacement therapy, can be included in a CR treatment study.

Treatment

CR extract should be administered orally twice daily at a dose of 0.48 mg/kg daily.

Dose(s) and schedule(s) can be subjected to changes.

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1. A method for relieving or curing symptoms or diseases which are caused by estrogen deficiency, or which can be relieved or cured by administration of steroidal estrogen, in a mammal who suffers from breast cancer, or has a risk of recurrent breast cancer, or has a high risk of developing breast cancer,

the method comprising administering, to the mammal, a composition

- which has an estrogen-like effect, as evidenced by a capability of the composition of inducing uterine growth in an adult ovariectomized female rodent, and
- which is free from interaction with breast cancer cells, in particular free from stimulating effect on breast cancer ,

thereby treating the estrogen deficiency-conditioned symptom or disease without introducing a risk of provoking the development of clinically evident breast cancer and/or stimulating growth of existing breast cancer cells in the mammal.

2. A method according to claim 1, wherein the mammal is female mammal.

3. A method according to claim 2, wherein the female mammal is a woman.

4. A method according to any of the preceding claims, wherein the estrogen-like effect possessed by the composition manifests itself in the composition being capable of inducing an increase in uterine weight in adult ovariectomized NMRI female athymic nude mice.

5. A method according to claim 4, wherein the increase in uterine weight following a dose comparable to a normal dose for the mammal to be treated corresponds to a weight increase seen in the same test animal following estradiol treatment.

6. A method according to claim 5, wherein the increase in uterine weight following a dose comparable to a normal dose for the mammal to be treated corresponds to a substantially maximum weight increase obtainable in the same test animal by estrogen treatment.

6. A method according to any of the preceding claims, wherein the composition is one which has no effect on the growth of estrogen receptor-negative cancer cells.

7. A method according to claim 6, wherein the composition is one which has no effect on the growth of xenografts of the estrogen and progesteron receptor-negative MDA-MB-231 human breast cancer cell line in nude mice.

8. A method according to any of the preceding claims, wherein the composition is one which is free from any effect on breast cancer cells even where the breast cancer cells are documented as being estrogen receptor-positive.

9. A method according to claim 8, wherein the composition is one which substantially does not bind to estrogen receptors of cancer cells.

10. A method according to any of the preceding claims, wherein the composition is one which has substantially no agonizing and substantially no antagonizing effect on the effect of estrogen such as estradiol on breast cancer cells, even where the breast cancer cells are documented as being estrogen receptor-positive.

11. A method according to any of claims 8-10, wherein the composition is one which has no effect on xenografts of the estrogen receptor-positive and estrogen dependent MCF-7 human breast cancer cell line in nude mice, as evidenced by the composition having no growth supportive effect and no growth inhibitory effect on the xenografts whether given alone or in combination with estradiol.

12. A method according to claim 11, wherein the composition is one which has no effect on xenografts of the estrogen receptor-positive and estrogen dependent MCF-7 human breast cancer cell line in nude mice, as evidenced by the composition having no growth supportive effect and no growth inhibitory effect on the xenografts whether given alone or in combination with estradiol, even where the composition is administered in a dose which is 10 or even 100 times higher than a dose giving, in the same strain of nude mice, a maximum uterus weight increase.

13. A method according to any of the preceding claims, wherein the estrogen deficiency-conditioned symptom or disease is selected from the group consisting of

menopausal symptoms, osteoporosis, osteochondrosis, hyperlipidaemia, hypercholesterolaemia, and arteriosclerosis.

14. A method according to claim 13, wherein the estrogen deficiency-conditions symptoms are menopausal symptoms.

15. A method according to any of the preceding claims, wherein the composition is a composition containing substances contained in *Cimicifuga Racemosa* extract, or derivatives thereof.

16. A method according to claim 15, wherein the composition is or contains *Cimicifuga Racemosa* extract.

17. A method according to claim 15, wherein the composition is a composition comprising *Cimicifuga Racemosa* plant parts.

17. A method according to claim 16, wherein the composition is a composition containing one or more chemical compounds contained in *Cimicifuga Racemosa* extract, or derivatives thereof.

18. A method for screening for substances or compositions which can be used in the method according to claim 1, comprising subjecting test substances or compositions to
1) testing for possible estrogen-like effect in normal tissue by measuring increase in uterine weight in an adult ovariectomized female rodent and
2) testing for possible estrogenic effect in breast cancer,
and selecting, as candidates for tissue-selective estrogenic substances or compositions useful in the method according to claim 1, substances or compositions which,
a) are capable of inducing uterine growth in an adult ovariectomized female rodent, and at the same time
b) have no effect on the growth of estrogen receptor-negative cancer cells and no effect on estrogen receptor-positive cancer cells in the doses in which they induce uterine growth.

19. A method according to claim 18, wherein the capability of the substance or composition of inducing uterine growth in an adult ovariectomized female rodent is tested by testing the capability of the substance or composition of effecting uterine weight increase in ovariectomized female NMRI athymic nude mice, the lack of effect of the substance or composition on the growth of estrogen receptor-negative cancer cells is assessed as the lack of capability of the substance or composition of supporting growth of MDA-MB231 xenografts in female NMRI athymic nude mice, and the lack of effect of the substance or composition on the growth of estrogen receptor-positive cancer cells is assessed as the lack of capability of the substance or composition of supporting growth of MCF-7 xenografts in female NMRI athymic nude mice.

Table 1

Individual uterine weights (g)		
Untreated controls	Estradiol (E_2)	CR
0.09	0.11	0.14
0.11	0.15	0.11
0.08	0.14	0.13
0.06	0.12	0.21
0.05	0.15	0.11
		0.12

Effect of CR on uterine weight of ovariectomized adult NMRI/Bom™ female athymic nude mice.
CR was given orally, 0.24 mg/kg twice dail. E2 treatment was given by subcutaneous inoculation of a 0.72 mg slow release pellet (inovative Research) in the neck.

Table 2

Group	Growth curve analysis				
	Tumors [1]	Gompertz growth curve parameters [2]			T_D [3]
	No	$\alpha \times 10^3$	$\beta \times 10^3$	r	days
Controls	15	16.5	96.3	0.992	13.0
CR Treated	16	14.4	80.3	0.994	14.9

Growth analysis of human breast carcinoma xenografts MDA-MB-231 grown in CR treated and untreated control female META/Bom™ athymic nude mice.

1) Number of evaluable tumors; inocula without growth were excluded.

2) The data on tumor area (: product of two perpendicular measurements) were described according to a Gompertz function.

$$A(t) = A(0)\exp((1-\exp(-\alpha t))\beta/\alpha)$$

A(0) is the tumor area at the time of tumor cell inoculation, A(t) is the tumor area at time t after inoculation, and α and β are constants determining the course of the growth curves. By linear regression the growth data were fitted to a straight lined transformation of the Gompertz function,

$$\ln[\ln A(max) - \ln A(t)] = \ln(\beta/\alpha) - \alpha t$$

A(max) is the theoretical maximal tumor area, α represents the slope, and r is the coefficient of correlation.

3) Tumor volume doubling time, T_D was computed from the Gompertz parameters. Tumor volume was calculated from

$$V(t) = \Pi/6\, A^{3/2}\, k,$$

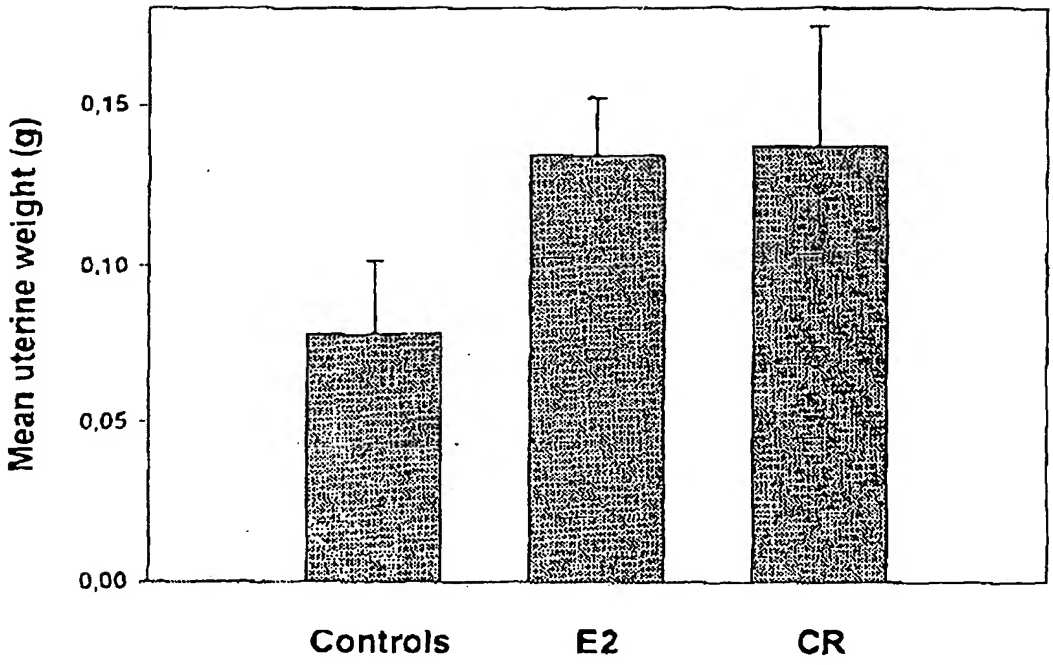
where A is tumor area, and k is a previously established constant of the relation between the two measurements obtained and the third dimension of the tumors.

Table 3

Group	Growth curve analysis				
	Tumors	Gompertz growth curve parameters			T_D
	No	$\alpha \times 10^3$	$\beta \times 10^3$	r	days
Estradiol (E2)	7/10	7.6	34.6	0.903	28.3
CR	0/10	-	-	-	-

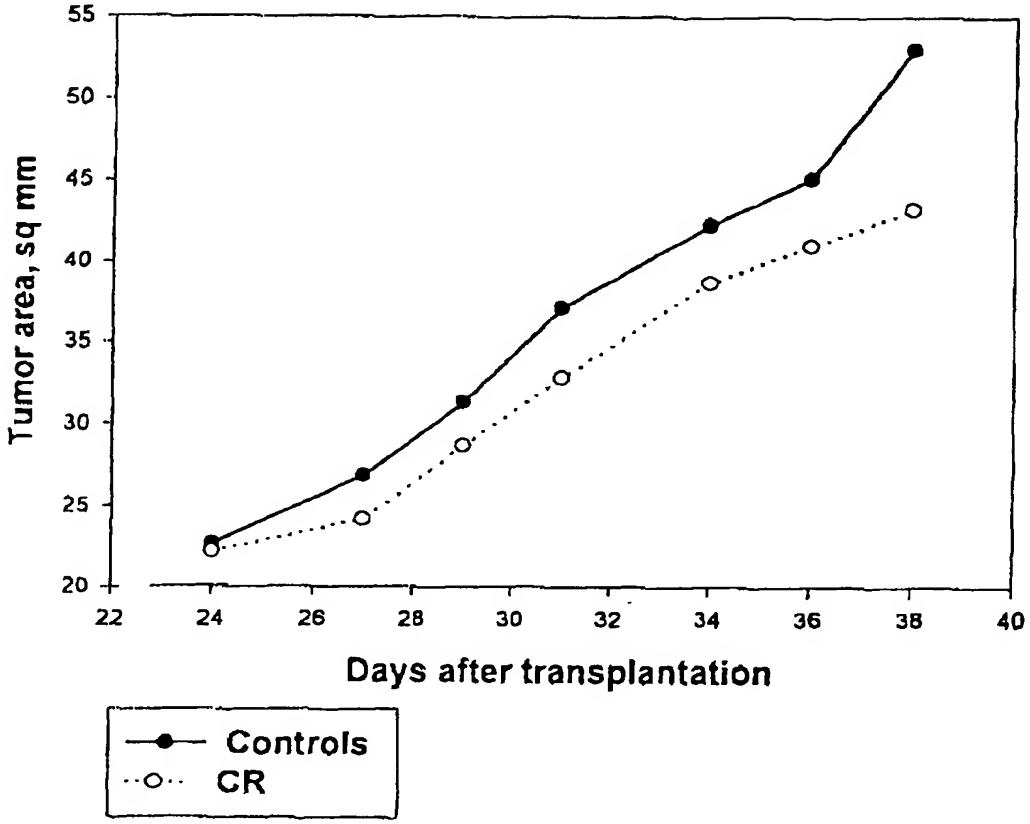
Growth analysis of human MCF-7 breast carcinoma xenografts grown in CR and E2 treated female NMRI/Bom™ athymic nude mice.

Figure 1



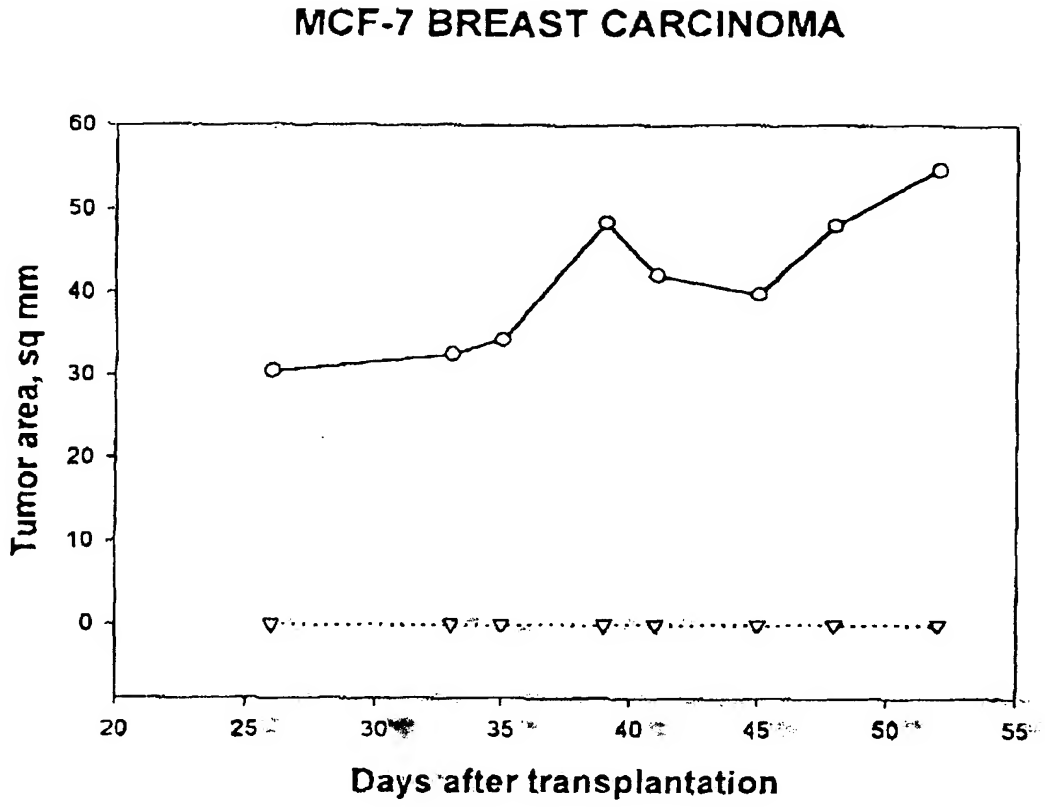
Effect of CR on mouse uterine weight

Figure 2



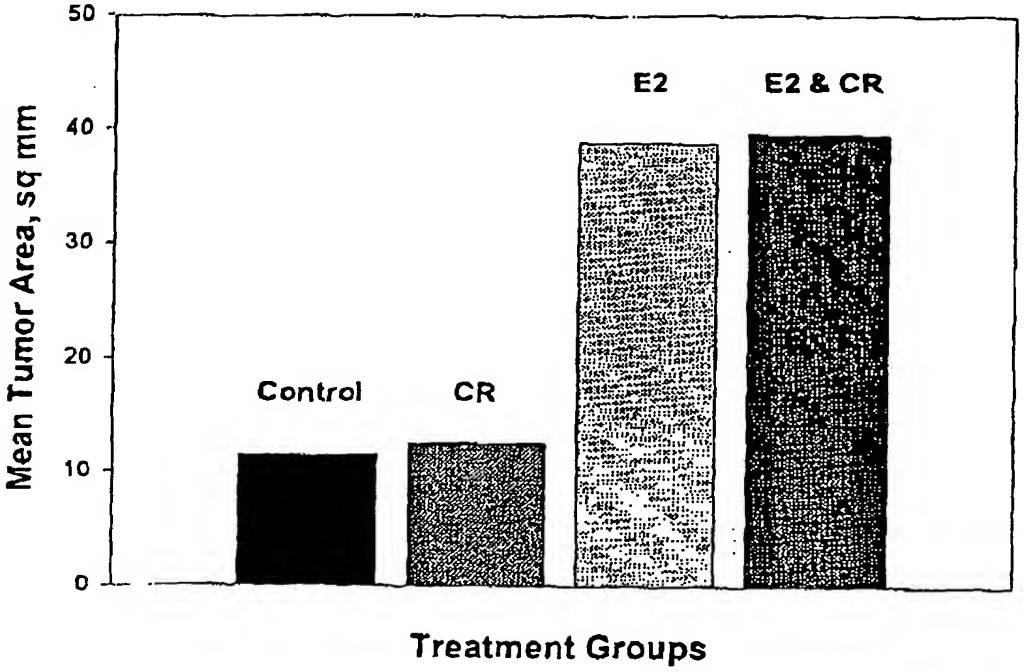
Effect of CR on the growth of MDA-MB-231 human breast cancer xenograft.

Figure 3



Mean tumor area growth curves of MCF-7 human breast carcinoma xenografts. The mice were treated with E2 or CR. The lack of tumor growth in CR treated mice are indicated in the figure.

Figure 4



Mean tumor size of MCF-7 xenografts in nude mice calculated from tumor measurements obtained at day 20 after transplantation. The calculations were based on 10, 5, 8, and 11 measurable tumors in the Control, CR, E2, and Combined treatment groups, respectively.